Flow Cytometric Determination of Cell Cycle Parameters of V79 Cells by Continuous Labeling with Bromodeoxyuridine

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ABSTRACT. A simple method with which to determine the cell cycle parameters, T_{G1} , T_S and T_{G2+M} (the durations of the G_1 , S and G_2+M phases) is described. V79 Chinese hamster lung cells were used to evaluate the method. After continuous labeling with bromodeoxyuridine (BrdU), V79 cells were stained with anti BrdU-monoclonal antibody with FITC (fluorescein isothiocyanate) and with PI (propidium iodide). The individual cells were checked by flow cytometry for green and red fluorescences whose signal intensities corresponded to the BrdU and cellular DNA contents. The durations of G_1 , S and G_2+M phases of V79 cells were determined by measuring the cell fractions containing the nonlabeled G_1 , labeled S and nonlabeled G_2+M phases. The reliability of this method is discussed.

Many methods have been developed to determine the cell cycle parameters, T_{G1} , T_S and T_{G2+M} (the durations of the G_1 , S and G_2+M phases) in a cell population growing exponentially (2, 7, 15, 21, 22, 25, 27, 28). In the cell cycle analysis method, the values of these cell cycle parameters in a cell population apparently are not affected by the measurement probes used.

Flow cytometry (20) has proven a reliable technique for the rapid measurement of kinetic parameters in a large cell population with minor perturbation to the cells (29). The simplest flow cytometric method for cell cycle analysis may be the method by which the T_{G1} , T_S and T_{G2+M} values in a cell population growing exponentially are determined from the ratio of cell fraction at each phase and from the duration of one cell cycle, T_c , (16). In this method, however, the fraction at each phase tends to vary with the analytical method used in the DNA fluorescence histogram analysis, and progression of the cell cycle can not be checked directly.

An autoradiography technique that uses the uptake of [3H]-thymidine has been extensively favored to determine the duration of each cell phase, and it has little effect on the cell cycle. The labeled mitosis method, in which the duration of each phase is determined from periodic changes in the labeled mitosis fractions after pulse labeling with [3H]-thymidine, also is often used. The RCS_i method (radioactivity per cell in a narrow window, S_i , in S phase) of cell cycle analysis was developed by Gray *et al.* (15) and is a combination of flow cytometry and autoradiography. But, although autoradiography is useful and reliable, more than a week is required to obtain results.

Recently, an anti-bromodeoxyuridine monoclonal antibody has been developed

(14) and used in flow cytometry (5, 6). In this technique, cells are doubly stained, with FITC for BrdU and with PI for DNA. When the stained cells are irradiated by a laser at 488 nm (the excitation light), the relative intensity of the green and red fluorescence present in the individual cells corresponds to their cellular BrdU and DNA contents. With this method, differential counting of BrdU-labeled cells, non-labeled G_1 cells and nonlabeled G_2+M phase cells can be done satisfactorily (13).

We here introduce a method to determine the cell cycle parameters in a cell population growing exponentially. V79 cells were used to check the method because of their short cell cycle. Changes in the fractions of the nonlabeled G_1 and nonlabeled G_2+M phase cells in a V79 cell population labeled continuously with BrdU were measured, and the duration of each phase in the cell cycle was determined. By this method, the T_{G1} and T_{G2M} values could be determined accurately, whereas the value of T_s varied; therefore, a simulation method was used to determine the T_s value.

MATERIALS AND METHODS

Cells. V79 Chinese hamster lung cells (line V79-B, obtained from Dr. Ishii, Osaka University) (11, 23) were maintained in a humidified atmosphere of 10% CO_2 at 37°C as growing monolayer cultures in Dulbecco-Vogt modified Eagle's medium containing 9% fetal bovine serum (FBS, HyClone, Sterile Systems Inc.). The culture was routinely subcultured at low density twice a week by splitting it at a ratio of about 1 : 50.

Cell preparation. V79 cells in logarithmic growth were plated in petri dishes (90 ϕ ; Falcon Plastics) at a density of 1×10^4 cells per dish. BrdU (Sigma) then was added at a final concentration of 10 μ M at time $t=0$, and the dishes cultured in a CO_2 incubator. At various intervals (Fig. 4) cells were washed with PBS⁽⁻⁾ (divalent cation-free phosphate-buffered saline) then treated with 0.3 ml trypsin-EDTA (0.02% and 0.2 mM) for 5 min at room temperature (RT), after which they were suspended in 5 ml of medium containing 9% FBS. This suspension was centrifuged and the cells resuspended in PBS⁽⁻⁾. After being left for 30 min at 0°C, the cells were fixed with 40% ethanol at 0°C for 1 h then stored at 4°C until use.

Cell staining. The fixed V79 cells were suspended in PBS⁽⁻⁾ containing 0.25% RNase (Type II-A, Sigma) then incubated for 30 min at 37°C, after which they were treated with 2 N HCl for 45 min at 25°C then neutralized with 0.1 M $Na_2B_4O_7$ (pH 8.5). Treatment with HCl denatures the double strand of the DNA producing single strand DNA, thus permitting the reaction of the anti BrdU monoclonal antibody to BrdU to take place in the DNA chain. In this experiment, the length of HCl treatment was made relatively long in order to detect, as accurately as possible, the incorporated BrdU molecules. About 10^6 cells were suspended in 50 μ l PBS⁽⁻⁾ containing 0.5% Tween 20, 2% BSA (bovine serum albumin) and 1 μ l anti-BrdU monoclonal antibody (Becton Dickinson) then incubated for 1 h at RT. After these cells had been washed 3 times with PBS⁽⁻⁾, they were stained with FITC-conjugated goat anti mouse IgG (Miles-Yeda Ltd.) to BrdU for 1 h at RT.

The stained cells were washed twice with PBS⁽⁻⁾ then stained with PI solution (50 μ g PI/1 ml PBS⁽⁻⁾) to determine the total DNA present. The doubly stained cells were checked in a flow cytometer for their green and red fluorescence as described below.

Flow cytometric measurements. The green and red fluorescence of individual V79 cells was measured with a Cytofluorograf system 50H (Ortho Instruments) equipped with a 5W argon ion laser. The fluorescence from individual cells irradiated with a focused laser beam at 488 nm (200 mW laser power) as the excitation light was separated optically through an

interference filter (530 nm peak and 20 nm band width) and a cut off filter (50% transmittance at 635 nm) and detected by photomultiplier tubes. The differential counting of the G_1 , S and G_2+M phase cells in a cell population was done visually through electronic windows for green and red fluorescence. The fractions of the G_1 , S and G_2+M phase cells were determined directly by counting the signals through the respective windows.

RESULTS

(A) *Analytical equations.* Cells in an asynchronous cell population are believed to traverse the cell cycle at a constant rate; *i.e.*, the cell number increases exponentially with the time, t . Therefore, let $n(t, \tau)$ be the number of cells per unit time at time t following through a point in the cell cycle which precedes division by time τ . $n(t, \tau)$ can be written (25, 22)

$$n(t, \tau) = \alpha N_0 \exp(\alpha t) \exp(\alpha \tau), \quad (1)$$

$$\alpha = \ln 2 / T_c \quad (2)$$

in which N_0 is the initial number of cells in mitosis and T_c is the length of the cell cycle.

If it is assumed that S phase cells incorporate BrdU as soon as it is added and that the progression of the cell cycle is not affected by its presence, the fractions representing the BrdU-labeled cells (F_1), nonlabeled G_1 cells (F_{G1}) and nonlabeled G_2+M cells (F_{G2M}) theoretically can be derived as functions of time t using Eqs. (1) and (2), as shown in Table 1. In Table 1, approximated equations also are given to facilitate the analysis. In practice, cell cycle parameters were determined using these approximate equations.

(B) *Analysis of the cell cycle of V79 cells.* To determine the cell cycle parameters, T_{G1} , T_S and T_{G2M} , V79 cells were labeled continuously with BrdU, then stained with PI for cellular DNA and with FITC for BrdU, after which the individual green and red fluorescence were measured by flow cytometry. At various times, the fractions containing BrdU-labeled cells, $F_1(t)$, nonlabeled G_1 phase cells, $F_{G1}(t)$ and nonlabeled G_2+M phase cells, $F_{G2M}(t)$ were measured.

TABLE 1. THEORETICAL EXPRESSION OF $F_1(t)$, $F_{G1}(t)$ AND $F_{G2M}(t)$

Theoretical expression		Approximate expression $F(t)=at + b$	
		a	b
$F_1(t)$			
t_1	$-\exp\{a(T_{G2M}-t)\}+2\exp(-aT_{G1})$	$\{\exp(aT_{G2M})-1\}/T_{G2M}$	$2\exp(-aT_{G1})-\exp(aT_{G2M})$
t_2	$-2\exp\{a(T_{G2M}-t)\}+1+2\exp(-aT_{G1})$	$\{2-2\exp(-aT_{G1})\}/T_{G1}$	$1-\{2-2\exp(-aT_{G1})\}(T_{G2M}+T_{G1})/T_{G1}$
$F_{G1}(t)$			
t_1	$2-2\exp(-aT_{G1})$	0	$2-2\exp(-aT_{G1})$
t_2	$2\exp\{a(T_{G2M}-t)\}-2\exp(-aT_{G1})$	$-\{2-2\exp(-aT_{G1})\}/T_{G1}$	$\{2-2\exp(-aT_{G1})\}(T_{G2M}+T_{G1})/T_{G1}$
$F_{G2M}(t)$			
t_1	$\exp\{a(T_{G2M}-t)\}-1$	$-\{\exp(aT_{G2M})-1\}/T_{G2M}$	$\exp(aT_{G2M})-1$

$F_1(t)$, $F_{G1}(t)$ and $F_{G2M}(t)$ are the fractions of BrdU-labeled cells, nonlabeled G_1 cells and nonlabeled G_2+M cells at time t . T_{G1} , T_S and T_{G2M} are the durations of the G_1 , S and G_2+M phases. α equals $\ln 2 / T_c$ and $T_c = T_{G1} + T_S + T_{G2M}$. t_1 and t_2 are the times when $0 \leq t_1 \leq T_{G2M}$ and $T_{G2M} \leq t_2 \leq T_{G2M} + T_{G1}$.

A cytogram (upper panel) of the green fluorescence relative intensity (F_{530})-red fluorescence relative intensity ($F_{>600}$) 10 min after the BrdU addition and its schematic pattern (lower panel) are shown in Fig. 1. Each dot represents a cell. In the upper panel, the lower left population is that of the G_1 phase cells, the lower right that of the G_2+M phase cells and the other that of the S phase cells. In the lower panel, the three rectangles with the symbols G_1 , S and G_2+M represent the counting windows for nonlabeled G_1 cells, labeled cells and nonlabeled G_2+M cells. Another window, T, which included all three windows was set for counting the total number of cells. These windows were set visually. The cell fractions passing through the G_1 , S and G_2+M windows were determined from the ratio of the cell number shown in each window to the number shown in the T window.

The change in the cytograms after the addition of BrdU is shown in Fig. 2. Careful inspection of this figure shows that the nonlabeled G_2+M and G_1 phase cells dis-

Fig. 1. F_{530} - $F_{>600}$ cytogram (A) and the schematic pattern (B). Rectangles labeled with G_1 , S and G_2+M represent the differential counting windows for the G_1 , S and G_2+M phase cells. The four angles represent the corners of the T window used to count the total cell number.

Fig. 2. Changes in $F_{530}-F_{600}$ cytograms with time after a BrdU addition. Numerals in each cytogram represent the incubation time (h) after the BrdU addition.

appear at 3 h and 6 h after the addition.

The affect of BrdU on the progression of V79 cells through the cell cycle was examined by measuring the changes in the DNA fluorescence histogram patterns after the BrdU addition. Red fluorescence histograms at 0, 0.5 and 12 h after the addition are shown in Fig. 3. These histograms were assayed for the G_1 , S and G_2+M phase fractions by cell cycle analysis (12, 13). Although no marked changes in these fractions were observed, a slight difference was found in the DNA distribution in the S phase. The late S phase fraction becomes low on the histogram 12 h after the BrdU addition. Note that the histograms in Fig. 3 are normalized for peak height.

Changes with time after BrdU addition in the fractions of labeled cells, nonlabeled G_1 cells, nonlabeled G_2+M cells and the total cells were counted through the respective windows shown in Fig. 1. Changes in the experimental values of $F_1(t)$, $F_{G1}(t)$ and $F_{G2M}(t)$ are shown in Fig. 4. T_{G1} and T_{G2M} values were determined accurately with an error of less than 30 min, from the times when $F_{G1}(t)$ and $F_{G2M}(t)$ became zero. Because the T_S value, in contrast, must be determined from the slope and/or the cell fraction at $t=0$, it was taken from simulation curves in which the T_c values were varied and the T_{G1} and T_{G2M} values fixed as described. Solid lines (A), (B) and (C) represent the simulation curves of $T_c=11$, 13 and 15 h when $T_{G1}=3.4$ and $T_{G2M}=2.4$ h. The duration of T_{G1} , T_S and T_{G2M} were determined as 3.4 ± 0.5 , 7.2 ± 2.0 and 2.4 ± 0.5 h.

Fig. 3. Changes in $F_{>600}$ frequency distribution histograms with time after a BrdU addition. The numerals in each histogram gives the incubation time in hours after the BrdU addition.

DISCUSSION

There are many methods to determine the cell cycle parameters T_{G1} , T_S and T_{G2M} , for a cell population in balanced growth. The best method requires high accuracy for the values obtained, a short determination period and the reliability that the method itself will not alter the cell cycle parameter values.

DNA histogram analysis of flow cytometry is useful for determining the G_1 , S and G_2+M phase fractions in a cell population in balanced growth (1, 3, 4, 8, 9, 10, 12, 13, 19, 26). But, fractions obtained by cell cycle analysis may be somewhat inaccurate depending on the treatment of the G_1 -S and S- G_2 boundaries. In addition, the T_c value must be known in order to calculate T_{G1} , T_S and T_{G2M} from the fractions obtained.

The BrdU continuous labeling method described in this report has several advantages: The length of the measurement period is relatively short; only the length of $T_{G1}+T_{G2M}$ (or T_{G2M}) is required to determine all the cell cycle parameters. In addition, the G_1 , S and G_2+M phase fractions can be accurately determined from the sample at $t=0$. Also, progression of the cell cycle can be observed directly.

Although the values of T_{G1} and T_{G2M} were easily determined from the time when $F_{G1}(t)$ and $F_{G2M}(t)$ became zero, the T_S value had to be determined from simulation curves. The simulation method was used for two practical reasons; a small variation in the phase fraction was enlarged on the T_S value and the degree of the slope was correlated with the phase fractions.

The simulation curves were approximated by using the first order functions of t . Approximation curves (solid lines) and exact ones (dotted lines) are shown in Fig. 5.

Fig. 4. Changes in the fractions of labeled cells (●), nonlabeled G_1 cells (○) and nonlabeled G_2+M cells (●) with incubation time after a BrdU addition. The abscissa gives the incubation time in hours. The left ordinate shows fractions actually observed. The right ordinate shows cell fractions corrected by considering noncycling cells. The solid lines (A), (B) and (C) are the simulation curves of $T_c=11, 13$ and 15 h, when T_{G_1} and T_{G_2M} were fixed as 2.4 and 3.4 h. Each circle represents the mean of ten measurements. Duplicate measurements were made, the results at particular times being combined with the longitudinal bar.

It is clear that any differences between the approximate and exact curves fall within the experimental error of measurement.

It is necessary to discuss the affect of BrdU on the progression of the cell cycle because BrdU is known to interfere with the advancement of some cycles (17, 18, 24). Fortunately, there was no marked effect of BrdU on the growth rate and cell phase fractions of the V79 cell population used. Recently we observed that B16 melanoma cells growing in MEM (minimal essential medium) supplemented with 2% FBS were affected by BrdU in concentrations above $0.5 \mu M$ (unpublished data). The effect of BrdU may vary with the growth conditions (*e.g.* the concentration of serum) and with the characteristics of the cells such as the amount of endogenous thymidine in the cells, the activities of kinases and deaminases and the functioning of alternate synthetic pathways. Serious effects of BrdU on a cell cycle would exclude use of any method in which cell cycle parameter values are determined from the cell cycle progression of BrdU-labeled cells, as is the case with the RCS_i method (15).

Nonlabeled cells disappeared in the cytograms at 6, 7 and 8 h (Fig. 2), which means that all the cells in the cell population advanced in the cycle and took up BrdU in the S phase. There is a possibility that cells containing BrdU change their cell cycle character after the BrdU uptake. It will be sure that BrdU is incorporated in

Fig. 5. Theoretical (broken lines) and approximate (solid lines) curves of $F_I(t)$, $F_{G1}(t)$ and $F_{G2M}(t)$. T_{G1} , T_S and T_{G2M} values were fixed to 3.4, 7.2, and 2.4 h.

S phase to DNA and that nonlabeled cells advance normally in the cycle. In our continuous BrdU-labeling method, therefore, the results are not affected by the presence of BrdU because the values of cell cycle parameters are determined by the times when the nonlabeled G_1 and G_2+M phase cell fractions disappear and by the fractions of the G_1 , S and G_2+M phase cells at time, $t=0$.

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